

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
14 April 2005 (14.04.2005)

PCT

(10) International Publication Number  
**WO 2005/033319 A2**

(51) International Patent Classification<sup>7</sup>: **C12N 15/82**

(21) International Application Number:  
PCT/US2004/032191

(22) International Filing Date: 1 October 2004 (01.10.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/508,409 2 October 2003 (02.10.2003) US

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(81) Designated States (*unless otherwise indicated, for every  
kind of national protection available*): AE, AG, AL, AM,

AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,  
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,  
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,  
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,  
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,  
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,  
ZW.

(84) Designated States (*unless otherwise indicated, for every  
kind of regional protection available*): ARIPO (BW, GH,  
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,  
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,  
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,  
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,  
GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished  
upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: **STACKING CROP IMPROVEMENT TRAITS IN TRANSGENIC PLANTS**

Amino Acid Sequence Alignment of CCAAT Box DNA Binding Transcription Factors

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SEQ ID NO:2 MAEAPASPGG GGGSHESGSP RGGGGGGSVR EQDRFLPIAN ISRIMKKAIP ANGKTIPANG
SEQ ID NO:3 MAEAPASPGG GGGSHESGSP RGGGGGGSVR EQDRFLPIAN ISRIMKKAIP ANGK-----
SEQ ID NO:6 MSDAPPSPFH --ESGGEQSP RG-SSSG-AR EQDRYLPIAN ISRIMKKALP PNGK-----
SEQ ID NO:7 MADTPSSPAG DGG---ES-- -----GGSVR EQDRYLPIAN ISRIMKKALP PNGK-----
SEQ ID NO:8                                     R EQDRYLPIAN ISRIMKKALP PNGKXXXXXX
SEQ ID NO:10MXXXPXSPPX
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SEQ ID NO:2 KIAKDAKETV QECVSEFISF ITSEASDKCQ REKRKTINGD DLLWAMATLG FEDYIEPLKV
SEQ ID NO:3 -IAKDAKETV QECVSEFISF ITSEASDKCQ REKRKTINGD DLLWAMATLG FEDYIEPLKV
SEQ ID NO:6 -IAKDAKDTM QECVSEFISF ITSEASEKCQ KEKRKTINGD DLLWAMATLG FEDYIEPLKV
SEQ ID NO:7 -IGKDAKDTV QECVSEFISF ITSEASDKCQ KEKRKTVNGD DLLWAMATLG FEDYLEPLKI
SEQ ID NO:8 XIAKDAKXTX QECVSEFISF ITSEASXKCQ XEKRKTINGD DLLWAMATLG FEDYIEPLKV
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SEQ ID NO:2 YLQKYREMEG DSKLTAKSSD GSIKKDALGH VGASSSAAQG MGQQGAYNQG MGYMQPQYHN
SEQ ID NO:3 YLQKYREMEG DSKLTAKSSD GSIKKDALGH VGASSSAAEG MGQQGAYNQG MGYMQPQYHN
SEQ ID NO:6 YLARYREAEG DTKGSARSGD GSATPDQVGL AGQNSQLVHQ GSLNYIGLQV ----QPQHLV
SEQ ID NO:7 YLARYRELEG DNKGSKGSGD GSNRDAGGGV SGEEMPSW
SEQ ID NO:8 YLXXYREXEG
SEQ ID NO:9 DSKLTAKSSD GSIKKDALGH VGASSSAAAG MGQQGAYNQG MGYMQPQYHN
```

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SEQ ID NO:2 GDISN
SEQ ID NO:3 GDISN
SEQ ID NO:6 MPSMQSHE
SEQ ID NO:9 GDISN
```

(57) Abstract: Seed for growing transgenic plants with a crop improvement trait produced by inserting into the genome of a pro-  
genitor plant a recombinant DNA construct which confers at least one crop improvement trait and, optionally, an herbicide resistance  
trait. Hybrid corn seed is produced by crossing a transgenic female ancestor corn plant having in its genome recombinant DNA  
which confers a crop improvement trait with a transgenic male ancestor corn plant having in its genome a recombinant DNA which  
confers at least one of an herbicide resistance trait of a pest resistance trait.



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## **Stacking Crop Improvement Traits in Transgenic Plants**

### **Incorporation of Sequence Listing**

The sequence listing is identical to the sequence listing submitted in provisional application No. 60/463,787, for Water-Deficit-tolerant Transgenic Plants (docket No. 38-  
5 21(52578)B, where the computer readable form was in the file named "ZMCAAT-2.ST25.txt" which is 14 kilobytes (measured in MS-Windows), was created on April 17, 2003, was submitted on a 3.5" diskette, and is incorporated herein by reference.

### **Cross Reference to Related Application**

This application claims benefit under 35 U.S.C. 119(e) of the United States Provisional  
10 Application Serial No. 60/508,409 filed 10/02/2003, which is herein incorporated by reference.

### **Field of the Invention**

Disclosed herein are DNA useful for producing transgenic plants and methods of using such DNA for producing transgenic plants and seed.

### **Background Of The Invention**

15 One of the goals of plant genetic engineering is to produce plants with agronomically, horticulturally or economically important traits including tolerance to any of a variety of environmental stresses. Many crop plants are transgenic comprising recombinant DNA that confers herbicide and/or pest resistance traits. Incorporation of additional recombinant DNA for conferring crop improvement traits in crop plants presents a challenge of using DNA constructs  
20 of increased complexity.

### **Summary of the Invention**

This invention provides transgenic plants with stacked engineered traits, e.g. a crop improvement trait in combination with herbicide and/or pest resistance traits. More particularly this invention provides seed for growing a transgenic plant with stacked engineered traits having  
25 in its genome a recombinant DNA construct for conferring a crop improvement trait and an herbicide resistance trait and/or a pest resistant trait.

Another aspect of the invention provides hybrid corn with stacked engineered traits. Such hybrid corn is the progeny of a transgenic female ancestor corn plant having in its genome a recombinant DNA which confers a crop improvement trait in combination with an herbicide  
30 and/or pest resistance trait. Embodiments of such hybrid corn have a transgenic male ancestor

corn plant which has in its genome recombinant DNA which confers herbicide resistance and/or pest resistance.

### Brief Description Of The Drawings

Figure 1 is an amino acid sequence alignment.

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### Detailed Description Of The Invention

SEQ ID NO: 1 provides the DNA sequence for an exogenous transcriptional unit comprising promoter elements, DNA encoding a *Zea mays* transcription factor and terminator elements.

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SEQ ID NO:2 provides the amino acid sequence of a transcription factor encoded by the DNA encoding a *Zea mays* transcription factor within the exogenous transcriptional unit of SEQ ID NO:1. The transcription factor is described as a CCAAT-box DNA binding protein subunit B.

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SEQ ID NO:3 provides the amino acid sequence for a *Zea mays* transcription factor that is homologous to the transcription factor with the amino acid sequence of SEQ ID NO:2. The transcription factor of SEQ ID NO:3 was originally disclosed by Li *et al.* in Nucleic Acid Res. 20(5), 1087-1091 (1992).

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SEQ ID NO:4 provides the DNA sequence of a *Zea mays* gene that encodes the transcription factor of SEQ ID NO:3.

SEQ ID NO:5 provides DNA sequence of a *Glycine max* gene that encodes a transcription factor that is homologous to the *Zea mays* transcription factors of SEQ ID NO:2 and 3.

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SEQ ID NO:6 provides the amino acid sequence for the a *Glycine max* transcription factor that is encoded by the DNA of SEQ ID NO:5.

SEQ ID NO:7 provides the amino acid sequence of an *Arabidopsis thaliana* transcription factor that is homologous to the *Zea mays* transcription factors of SEQ ID NO:2 and 3.

SEQ ID NO:8 is an artificial consensus amino acid sequence of a common core region of amino acids of the transcription factors of SEQ ID NO:2, 3, 6 and 7.

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SEQ ID NO:9 is an artificial consensus amino acid sequence of a common terminus region of the *Zea mays* transcription factors of SEQ ID NO:2 and 3.

SEQ ID NO:10 is an artificial consensus amino acid sequence of a common terminus region of eight amino acids of the transcription factors of SEQ ID NO:2, 3, 6 and 7.

As used herein “crop improvement trait” means a trait that produces a crop yield or quality improvement, e.g. yield, *per se*, such as increased seed number and/or increased seed weight; tolerance to an environmental stress such as cold, heat, plant density or water deficiency; tolerance to a nutrient deficiency such a nitrogen or phosphorus deficiency; a plant growth and development improvement such as decreased abscission, delayed vegetative senescence, early maturity, increased plant size, increased plant growth rate, increased biomass, enhanced photosynthesis under low light and increased fertility; improved seed quality such as decreased ovule abortion, enhanced endosperm cell number or size, enhanced number or size of leaf or seed starch granules and increased oil, protein or starch accumulation; improved source efficiency such as improved photosynthesis efficiency, increased sugar accumulation, increases starch accumulation, increased sugar export, increases carbon and nitrogen partitioning and increased amino acid transport; improved crop quality such as an increase in one or more amino acids, an increase in seed carbohydrate, protein or oil levels and an increase in total seed oil and protein levels.

As used herein “water deficit” is a plant condition characterized by water potential in a plant tissue of less than  $-0.7$  megapascals (MPa), e.g.  $-0.8$  Mpa. Water potential in maize is conveniently measured by clamping a leaf segment in a pressurizable container so that a cut cross section of leaf is open to atmospheric pressure. Gauge pressure (above atmospheric pressure) on the contained leaf section is increased until water begins to exude from the atmospheric-pressure-exposed cross section; the gauge pressure at incipient water exudation is reported as negative water potential in the plant tissue, e.g. 7 bars of gauge pressure is reported as  $-0.7$  MPa water potential. Water deficit can be induced by withholding water from plants for sufficient time that wild type plants are deleteriously affected, e.g. as manifested by reduced yield, stunted growth, retarded development, death or the like. The plants of this invention show a remarkable risibility after periods of water deficit that are adverse to wild type plants.

As used herein “yield” of a crop plant means the production of a crop, e.g. shelled corn kernels or soybean or cotton fiber, per unit of production area, e.g. in bushels per acre or metric tons per hectare, often reported on a moisture adjusted basis, e.g. corn is typically reported at 15.5 % moisture. Moreover a bushel of corn is defined by law in the State of Iowa as 56 pounds

by weight, a useful conversion factor for corn yield is: 100 bushels per acre is equivalent to 6.272 metric tons per hectare. Other measurements for yield are in common practice.

As used herein a “transgenic” organism, e.g. plant or seed, is one whose genome has been altered by the incorporation of a trait-conferring, recombinant DNA, e.g. exogenous DNA or additional copies of native DNA, e.g. by transformation or by breeding with a transformed plant. Thus, transgenic plants include progeny plants of an original plant derived from a transformation process including progeny of breeding transgenic plants with wild type plants or other transgenic plants. The enhancement of a desired trait can be measured by comparing the trait property in a transgenic plant which has recombinant DNA conferring the trait to the trait level in a progenitor plant. As used herein “progenitor plant” refers to a plant of essentially the same genotype as a transgenic plant but lacking the specific trait-conferring, recombinant DNA that characterizes the transgenic plant. Such a progenitor plant that lacks that recombinant DNA can be a natural, wild-type plant, an elite, non-transgenic plant, or a transgenic plant without the specific trait-conferring, recombinant DNA that characterizes the transgenic plant. The progenitor plant lacking the specific, trait-conferring recombinant DNA can be a sibling of a transgenic plant having the specific, trait-conferring recombinant DNA. Such a progenitor sibling plant may comprise other recombinant DNA.

Crop plants of interest in the present invention include, but are not limited to, soybean (including the variety known as *Glycine max*), cotton, canola (also known as rape), corn (also known as maize and *Zea mays*), wheat, sunflower, sorghum, alfalfa, barley, millet, rice, tobacco, fruit and vegetable crops and turfgrasses.

As used herein an “herbicide resistance” trait is a characteristic of a transgenic plant that is resistant to dosages of an herbicide that is typically lethal to a progenitor plant. Such herbicide resistance can arise from a natural mutation or more typically from incorporation of recombinant DNA that confers herbicide resistance. Herbicides for which resistance is useful in a plant include glyphosate herbicides, phosphinothricin herbicides, oxynil herbicides, imidazolinone herbicides, dinitroaniline herbicides, pyridine herbicides, sulfonylurea herbicides, bialaphos herbicides, sulfonamide herbicides and gluphosinate herbicides. To illustrate the that production of transgenic plants with herbicide resistance is a capability of those of ordinary skill in the art reference is made to U.S. patent application publications 2003/0106096A1 and

2002/0112260A1 and U.S. Patents 5,034,322; 6,107,549 and 6,376,754, all of which are incorporated herein by reference.

As used herein an “pest resistance” trait is a characteristic of a transgenic plant is resistant to attack from a plant pest such as a virus, a nematode, a larval insect or an adult insect that typically is capable of inflicting crop yield loss in a progenitor plant. Such pest resistance can arise from a natural mutation or more typically from incorporation of recombinant DNA that confers pest resistance. For insect resistance, such recombinant DNA can, for example, encode an insect lethal protein such as a delta endotoxin of *Bacillus thuringiensis* bacteria or be transcribed to a dsRNA targeted for suppression of an essential gene in the insect. To illustrate that the production of transgenic plants with pest resistance is a capability of those of ordinary skill in the art reference is made to U.S. Patents 5,250,515 and 5,880,275 which disclose plants expressing an endotoxin of *Bacillus thuringiensis* bacteria, to U.S. Patent 6,506,599 which discloses control of invertebrates which feed on transgenic plants which express dsRNA for suppressing a target gene in the invertebrate, to U.S. Patent 5,986,175 which discloses the control of viral pests by transgenic plants which express viral replicase, and to U.S. Patent Application Publication 2003/0150017 A1 which discloses control of pests by a transgenic plant which express a dsRNA targeted to suppressing a gene in the pest, all of which are incorporated herein by reference.

**Protein and Polypeptide Molecules** - As used herein “protein” means a polypeptide of combined amino acids including a natural protein or polypeptide fragment of a natural protein or a modified natural protein or a synthetic protein, or a peptide having a protein function. Proteins produced and used by the transgenic plants of this invention are whole proteins or at least a sufficient portion of an entire protein to impart the relevant biological activity of the protein, e.g. a crop improvement trait. To illustrate, reference is made to Hap3 proteins which are effective in conferring water deficit tolerance in transgenic plants, e.g. when constitutively expressed. The Hap3 proteins are transcription factors having a CCAAT-box DNA binding domain and include the *Zea mays* transcription factors of SEQ ID NO: 2 and 3, the *Glycine max* transcription factor of SEQ IS NO:6 and the *Arabidopsis thaliana* transcription factor of SEQ ID NO:7 or a functionally-equivalent homologous transcription factor. Such functionally-equivalent homologous transcription factor can be defined by the consensus amino acid sequences that characterize these transcription factors. With reference to Figure 1 the defining consensus

sequences are the central amino acid region consensus sequence of SEQ ID NO:8 and at least one of the terminus amino acid consensus sequences of SEQ ID NO:9 and SEQ ID NO:10.

Aside from similarity in function homologs of proteins or DNA can be described as molecules having a sequence, e.g. amino acid or nucleotide sequence, which shares identity to a reference sequence. For simplicity, DNA homologs are defined by optimally aligning the nucleotide sequence of a putative DNA homolog with a defined nucleotide sequence and determining identical nucleotide elements over a window of the defined sequence. Similarly, protein homologs of a consensus amino acid sequence is defined by optimally aligning the amino acid sequence of a putative protein homolog with a defined amino acid sequence and determining the identical amino acid elements over a window of the defined sequence. Optimal alignment can be effected manually but more preferably with the assistance of a homology-based search algorithms such as those commonly known and referred to as BLAST, FASTA, and Smith-Waterman.

For other than consensus amino acid sequences protein homologs are defined by optimally aligning the amino acid sequence of a putative protein homolog with a defined amino acid sequence and determining the conservatively substituted amino acid elements over a window of the defined sequence. Conservatively substituting amino acids are (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; (4) neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; (5) amino acids having aliphatic side chains such as glycine, alanine, valine, leucine, and isoleucine; (6) amino acids having aliphatic-hydroxyl side chains such as serine and threonine; (7) amino acids having amide-containing side chains such as asparagine and glutamine; (8) amino acids having aromatic side chains such as phenylalanine, tyrosine, and tryptophan; (9) amino acids having basic side chains such as lysine, arginine, and histidine; (10) amino acids having sulfur-containing side chains such as cysteine and methionine. To account for insertions and deletions, mutations, variations in the C and/or N terminal regions of proteins and non-conservative substitutions, protein homologs are defined as being at least 80% identical.

**Recombinant DNA Constructs** - The present invention contemplates the use of polynucleotides which encode a protein effective for imparting resistance and/or tolerance to water deficit in plants. Such polynucleotides are assembled in recombinant DNA constructs using methods known to those of ordinary skill in the art. A useful technology for building DNA constructs and vectors for transformation is the GATEWAY™ cloning technology (available from Invitrogen Life Technologies, Carlsbad, California) uses the site specific recombinase LR cloning reaction of the Integrase/*att* system from bacteriophage lambda vector construction, instead of restriction endonucleases and ligases. The LR cloning reaction is disclosed in U.S. Patents 5,888,732 and 6,277,608, U.S. Patent Application Publications 2001283529, 2001282319 and 20020007051, all of which are incorporated herein by reference. The GATEWAY™ Cloning Technology Instruction Manual which is also supplied by Invitrogen also provides concise directions for routine cloning of any desired RNA into a vector comprising operable plant expression elements.

Recombinant DNA constructs used for transforming plant will comprise DNA cells for conferring a trait along with other commonly used DNA elements. As is well known in the art such constructs typically also comprise a promoter and other regulatory elements, 3' untranslated regions (such as polyadenylation sites), transit or signal peptides and marker genes elements as desired. For instance, see U.S. Patents No. 5,858,742 and 5,322,938 which disclose versions of the constitutive promoter derived from cauliflower mosaic virus (CaMV35S), U.S. Patent 6,437,217 which discloses a maize RS81 promoter, U.S. Patent 5,641,876 which discloses a rice actin promoter, U.S. Patent 6,426,446 which discloses a maize RS324 promoter, U.S. Patent 6,429,362 which discloses a maize PR-1 promoter, U.S. Patent 6,232,526 which discloses a maize A3 promoter, U.S. Patent 6,177,611 which discloses constitutive maize promoters, U.S. Patent 6,433,252 which discloses a maize L3 oleosin promoter, U.S. Patent 6,429,357 which discloses a rice actin 2 promoter and intron, U.S. Patent 5,837,848 which discloses a root specific promoter, U.S. Patent 6,084,089 which discloses cold inducible promoters, U.S. Patent 6,294,714 which discloses light inducible promoters, U.S. Patent 6,140,078 which discloses salt inducible promoters, U.S. Patent 6,252,138 which discloses pathogen inducible promoters, U.S. Patent 6,175,060 which discloses phosphorus deficiency inducible promoters, U.S. Patent Application Publication 2002/0192813A1 which discloses 5', 3' and intron elements useful in the design of effective plant expression vectors, U.S. patent application Serial No. 09/078,972



which discloses a coixin promoter, U.S. patent application Serial No. 09/757,089 which discloses a maize chloroplast aldolase promoter, all of which are incorporated herein by reference.

In many aspects of the invention it is preferred that the promoter element in the DNA construct should be capable of causing sufficient expression to result in the production of an effective amount of the transcription factor in water deficit conditions. Such promoters can be identified and isolated from the regulatory region of plant genes which are over expressed in water deficit conditions. Alternatively, such promoters can be exogenous constitutive promoters. Another class of useful promoters are water-deficit-inducible. Specific water-deficit-inducible promoters for use in this invention are derived from the 5' regulatory region of genes identified as a heat shock protein 17.5 gene (*HSP17.5*), an HVA22 gene (*HVA22*), and a cinnamic acid 4-hydroxylase (CA4H) gene (*CA4H*) of *Zea mays*; such water-deficit-inducible promoters are disclosed in U.S. provisional application Serial No. 60/435,987, filed December 20, 2002, incorporated herein by reference. Another water-deficit-inducible promoter is derived from the *rab-17* promoter as disclosed by Vilardell *et al.*, *Plant Molecular Biology*, 17(5):985-993, 1990.

In general it is preferred to introduce heterologous DNA randomly, i.e. at a non-specific location, in the plant genome. In special cases it may be useful to target heterologous DNA insertion in order to achieve site specific integration, e.g. to replace an existing gene in the genome. In some other cases it may be useful to target a heterologous DNA integration into the genome at a predetermined site from which it is known that gene expression occurs. Several site specific recombination systems exist which are known to function implants include cre-lox as disclosed in U.S. Patent 4,959,317 and FLP-FRT as disclosed in U.S. Patent 5,527,695, both incorporated herein by reference.

Constructs and vectors may also include a transit peptide for targeting of a gene target to a plant organelle, particularly to a chloroplast, leucoplast or other plastid organelle. For a description of the use of a chloroplast transit peptide see U.S. Patent 5,188,642, incorporated herein by reference.

In practice DNA is introduced into only a small percentage of target cells in any one transformation experiment. Marker genes are used to provide an efficient system for identification of those cells that are stably transformed by receiving and integrating a transgenic DNA construct into their genomes. Preferred marker genes provide selective markers which confer resistance to a selective agent, such as an antibiotic or herbicide. Any of the herbicides to

which plants of this invention may be resistant are useful agents for selective markers.

Potentially transformed cells are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene is integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm

5 stable integration of the exogenous DNA. Commonly used selective marker genes include those conferring resistance to antibiotics such as kanamycin (*nptII*), hygromycin B (*aph IV*) and gentamycin (*aac3* and *aacC4*) or resistance to herbicides such as glufosinate (*bar* or *pat*) and glyphosate (EPSPS). Examples of such selectable are illustrated in U.S. Patents 5,550,318; 5,633,435; 5,780,708 and 6,118,047, all of which are incorporated herein by reference.

10 Screenable markers which provide an ability to visually identify transformants can also be employed, *e.g.*, a gene expressing a colored or fluorescent protein such as a luciferase or green fluorescent protein (GFP) or a gene expressing a *beta*-glucuronidase or *uidA* gene (GUS) for which various chromogenic substrates are known.

**Crop improvement traits** – This invention employs recombinant DNA constructs in providing the transgenic seeds for growing a transgenic plant with a crop improvement trait. Such a transgenic plant has a crop improvement trait stacked with an herbicide resistance trait and/or a pest resistance trait. Such a transgenic plant has improved crop trait as compared to a progenitor plant not having recombinant DNA which codes for the crop improvement trait. Crop improvement traits include cold tolerance, improved nitrogen processing, improved phosphorus use efficiency, reduced abscission, early maturity, enhanced growth and leaf development, enhanced growth rate, low light tolerance, optimized C3 to C4 transition, improved plant development, increased plant size, population stress tolerance, reduced plant size, improved seed germination, delayed senescence, increased amino acid content, increased seed protein, increased seed oil, increased seed starch biosynthesis, increased endosperm cell number and/or size, increased vitamin content, increased photosynthesis efficiency, increased source enhancement, enhanced transport of amino acids, enhanced water deficit tolerance. Such crop improvement traits can provide yield improvement. For instance, when exposed to adverse water deficit conditions, plants with improved water deficit tolerance exhibit improved yield as compared to progenitor plants not having recombinant DNA which confers the water deficit tolerance trait.

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A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is cold tolerance, e.g. as indicated by increased plant recovery rate or plant survival rate after growth under freezing temperatures or increased germination and seedling vigor at temperature below 10 °C and/or cold and wet conditions. See table 1 for genes that can be useful for achieving cold tolerance, e.g. when over expressed in a recombinant DNA construct.

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Table 1

Cold tolerance genes	Reference
CBF transcription factors	Nature Biotechnology (1999) 17, 287-291
CBF1	Science. 1998 Apr 3;280(5360):104-6.
ICE1	Genes Dev. 2003 Apr 15;17(8):1043-54. Epub 2003 Apr 02.
CodA	Plant J 1998 Oct;16(2):155-61; Plant J 1997 Jul;12(1):133-42; Plant J 2000 Jun;22(5):449-53
GS2	Plant Mol Biol. 2000 May;43(1):103-11.
COR15a	Proc Natl Acad Sci U S A. 1996 Nov 12;93(23):13404-13409; Proc Natl Acad Sci U S A. 1998 Nov 24;95(24):14570-5.
FAD7; FAD8	Kodama H et al. (1994) Plant Physiol. 105(2): 601-605. Kodama et al. (1995) Plant Physiol. 107: 1177-85. Murakami et al. (2000) Science 287: 476-479). Bertin et al. (1998) Plant Growth Reg. 24: 31-41.
ABI3	Plant J. 2001 Jan;25(1):1-8.
CBF3	Plant Physiol. 2000 Dec;124(4):1854-65. Plant Cell. 2001 Jan;13(1):61-72.
OsCDPK7	Plant Cell Physiol. 2001 Nov;42(11):1228-33.
NtSCOF-1	Mol Cells. 2001 Oct 31;12(2):204-8 ; Plant J. 2001 Feb;25(3):247-59.
AtPP2CA	Plant J. 2001 May;26(4):461-70; J Exp Bot. 2001 Jan;52(354):181-2.
AtGST/GPX	Nat Biotechnol. 1997 Oct;15(10):988-91.
NtSOD	Plant Physiol. 1993 Dec;103(4):1067-1073.
pBE2113/hiC6	Biosci Biotechnol Biochem 2001 Aug;65(8):1796-804
OsWX	Mol Biol Evol. 1998 Aug;15(8):978-87.
ADH	Plant Physiol. 111: 381-391; Plant Physiol. 105: 1075-1087
AtHK1	FEBS Lett. 427: 175-178

Cold tolerance genes	Reference
ADS2	Plant Cell Physiol. 39: 247-253
RD29A	Plant Physiol. 103: 1047-1053 ; Plant Mol. Biol . 21: 641-653 ; Mol. Gen. Genet. 236: 331-340
XERO1	FEBS Lett. 381: 252-256
P5CS1	Plant J. 12: 557-569
P5CS2	Plant J. 12: 557-569
PAL	Plant Physiol. 108: 39-46
PDC1	Plant Physiol. 119: 599-607
PUMP	FEBS Letts. 429: 403-406
RCI3	Plant J. 2002 Oct;32(1):13-24.
NDPK2	Proc Natl Acad Sci U S A 2003 Jan 7;100(1):358-63
GPAT	Plant Cell Physiol. 2002 Jul;43(7):751-8; Physiol Plant. 2003 May;118(1):57-63.
AtCCT2	Plant Cell Physiology 0043 01342-01350; Mol Cells. 28;11(1):95-9; Mol Cells. 1997 Feb 28;7(1):58-63.
AZF1, AZF2, AZF3, STZ, GCN5	Gene 248:23-32, 2000.
Lipid desaturase	Nature Biotechnology (1996) 14, 1003-1006.
<i>AtSAC1</i>	Plant J 34, 293-306 (2003)

5 A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is nitrogen processing, e.g. the ability of a plant to fix or reduce nitrogen from N<sub>2</sub> into NO<sub>3</sub>, NH<sub>3</sub> or organic molecules. Nitrogen processing can also be characterized as low nitrogen tolerance as indicated by increased plant growth rate, biomass, seed set, photosynthesis under growth limiting nitrogen conditions. Nitrogen processing can also be characterized as enhanced nitrogen uptake,

translocation, storage or seed partitioning. See table 2 for genes that can be useful for nitrogen processing, e.g. when over expressed in a recombinant DNA construct.

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Table 2

Nitrogen processing	Reference
Nodulin-35 (uricase)	Plant J. 3: 599-606, 1993.
G225	US patent application Serial No. 10/407,920, incorporated herein by reference.
NDP kinase	PNAS (2003) 100: 358-363
iscN	Molecular Genetics Genomics (2002) 267: 820-828
HIUHase .	Plant Physiology 0130 02061-02068 2002.
Glutamine synthetase 2	Migge et al. (2000). Planta 210, 252-260
Nitrogenase in chloroplasts	Plant and Soil (1997) 193-203
Asparagine synthetase	Lam et al. (2003). Plant Physiol. 132, 926-935
AtUPS1	The Plant Cell (2002) 14: 847-856
AtDUR3	The Plant Cell (2003) 15: 790-800
AtNRT1;1	The Plant Cell (2003) 15: 107-117
OsAMT1;1 to OsAMT1;3	Plant Cell Physiol. (2003) 44(7):726-34.
asparagine synthase	several patents in this area
Glutamine synthetase 1	Oliveira et al. (2002). Plant Physiol. 129, 1170-1180

10 A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is improved phosphorus use efficiency, e.g. enhanced phosphorus uptake, translocation or vacuolar concentration. The tomato phosphate transporter gene (*LePT3*) as disclosed in International

Patent Publication WO0155299A1 can be useful for achieving improved phosphorus use efficiency in plants, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is reduced abscission, e.g. the absence of flower petal abscission, seed dehiscence/shatter or silique splitting. See table 3 for genes that can be useful for achieving reduced abscission in plants, e.g. when over expressed in a recombinant DNA construct.

Table 3

Reduced abscission	Reference
<i>ALC</i>	Curr. Biology 11, 1914-1922 (2001)
<i>HAESA</i>	Genes Dev.14(1):108-17 (2000)
<i>SHP1</i>	Nature 404; 766-770 ; 2000
<i>JOINTLESS</i>	Nature 406 910-913: 2000.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is early maturity, e.g. early or accelerated seed dry down or silique senescence with normal onset of flowering. *GPA1* and *OST1* genes with seed/husk specific expression as disclosed in J. Exp.Bot., 51, 447-458 and Science, 292:2070-2 and Trends Plant Sci., 8:151-3 can be useful for achieving early maturity in plants, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is enhanced growth and leaf development. The *Oshox1* gene as disclosed in Plant J., 11(2):263-76 can be useful for achieving enhanced growth and leaf development in plants, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is enhanced growth rate without causing significant premature flowering. The *PAUSED* gene as disclosed in Plant Physiol, 132(4):2135-43 (Aug. 2003) can be useful for achieving such enhanced growth rate in plants, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is low light tolerance, e.g. as characterized by plants which exhibit insensitivity to low light stress or altered spectral quality stress or enhanced photosynthesis under low light conditions. See

table 4 for genes that can be useful for achieving low light tolerance in plants, e.g. when over expressed in a recombinant DNA construct.

Table 4

Low light tolerance	Reference
ARR4.	Plant Physiology, 128: 363-369 (2002)
CRY2	Cell, 103:815-827
PHYB	Wagner et al., Plant Cell 3, 1275-1288; Short, Plant Physiol. 119, 1497-1505; Halliday et al., Plant J. 12, 1079-1090; Thiele et al., Plant Physiol. 120, 73-81; Jackson et al., Plant J. 9, 159-166

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is optimized C3 to C4 transition, e.g. as characterized by plants having an operational C4 pathway in C3 plants or the establishment of Kranz anatomy, which results in increased plant vigor or growth rate. See table 5 for genes that can be useful for achieving optimized C3 to C4 transition in plants, e.g. when over expressed in a recombinant DNA construct.

Table 5

Optimized C3 to C4 transition	Reference
C4PPDK	PNAS (1993) 90: 9586.
PEPC	Nat. Biotechn. (1999) 17:76.
C4 Phosphoenolpyruvate carboxylase	Plant Production Science (2001) 4: 311 - 312

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is improved plant development, e.g. as characterized by control of meristem patterning to increase the number of fertile flowers, number of fertilized ovules, organ number or yield. The *Blind*



gene as disclosed in PNAS, 99, 1064-1069 (2002) can be useful for achieving such improved plant development, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is improved plant growth, e.g. as indicated by cell expansion. The *UCU1* gene as disclosed in Dev.Biol., 242, 161-173 (2202) can be useful for achieving such improved plant growth, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is improved plant productivity, e.g. as characterized by increased root and shoot growth rates, increased biomass and harvest index, increased seed size and number, increased seed yield and/or increased seed oil content. See table 6 for genes that can be useful for achieving improved plant productivity, e.g. when over expressed in a recombinant DNA construct.

Table 6

Improved plant productivity	Reference
AGL8	US Patent 6,229,068 B1
ANT	US patent application publication 2002/0170093 A1

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased plant size, e.g. as characterized by increased cell size or cell number in organs such as seed, leaf, flower, roots, vascular bundles. See table 7 for genes that can be useful for achieving improved plant productivity, e.g. when over expressed in a recombinant DNA construct.

Table 7

Increased plant size	Reference
ANT	US patent application publication 2002/0170093 A1
ATHB6 (Transcription factor)	Plant Mol Biol. 1999 Aug;40(6):1073-83
4CL	Lee, D., et al., (1997) Plant Cell 9: 1985-1999

Increased plant size	Reference
ARGOS	Plant cell 15, 1951-1961 (2003)
<i>TFL1</i>	Dev.125, 1609-1615.(1998)
DWF4	Choe et al. (2001) Plant Journal 26: 573-582.
GA20 oxidase	Carrera et al., Plant J. 22, 247-256; Coles et al., Plant J. 17, 547-556
plant hemoglobin genes	Plant Journal (2003) 35: 763-770. PNAS (1998) 95: 10317 - 10321. Nature Biotech 15: 244-247

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is population stress tolerance, e.g. as characterized by increased and/or uniform biomass, root mass, growth rate, photosynthesis, seed set or yield under population stress. The *AtHB2* gene as disclosed in Development, 126:4235-4245 (1999) and the *PHYB* gene as disclosed in Nature Biotechnology 17, 287-291 (1999) can be useful for achieving population stress tolerance in plants, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is improved seed germination. The *DAG1* gene as disclosed in Plant physiology 128(2):411-7 and the *AtCYP51* gene as disclosed in Biochem Biophys Res Commun 6: 285(1):98-104 can be useful for achieving improved seed germination, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is short plants, e.g. as characterized by reduced internode length. The *CRY1* gene as disclosed in Plant Cell 13(12):2573-87, the *AtGA2ox8* gene as disclosed in Plant Cell 15(1):151-63 and the *GA-20 oxidase* gene as disclosed in Plant Physiol 126, 97-107 can be useful for achieving short plants, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is delayed senescence, e.g. as characterized by plants with delayed vegetative senescence together with extended photosynthesis and/or without a delay in the onset of flowering. See table 8 for

genes that can be useful for achieving delayed senescence in plants, e.g. when over expressed in a recombinant DNA construct.

Table 8

Delayed senescence	Reference
DWF4 expression in leaves	Plant Journal 26: 573-582.
KNOTTED 1	Plant Cell. 1999 Jun;11(6):1073-80.
ARR2	Nature, 413: 383-389.(2001)
G571	WO03014327A2
ORE9	Plant Cell 13(8):1779-90; 2001.
PRPS17/ORE4	Plant J. 31, 331-340; 2002.
AGL15	Plant Cell. 12:183-98; 2000.
PAO	Journal of Experimental Botany :53;801-808: 2002.
SAG13	PCT/US03/14989 US provisional application No. 60/381100
G1050	WO03014327A2
SAG promoter w/IPT gene	Science 270:1986-8

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased amino acid content in seed, e.g. without a decrease in protein or oil. The *CGSI* gene as disclosed in Plant physiology 128:97-107 can be useful for achieving improved seed germination, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased seed protein, e.g. without an reduction in oil components. The asparagine synthase gene can be useful for achieving increased seed protein, e.g. when over expressed in a recombinant DNA construct. See also US provisional application Serial No. 60/479,962, filed June 19, 2003, incorporated herein by reference, for other genes which are useful for increased seed protein when over expressed in a recombinant DAN construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased seed oil, e.g. without a reduction in protein components. The *AtNHX1* gene as disclosed in Proc.Nat..Acad.Sci.USA, 98,12832-12836 and genes disclosed in US application Serial No. 10/613,520, filed July 2, 2003, incorporated herein by reference, including pyruvate kinase and phosphofructokinase can be useful for achieving increased seed oil, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased vitamin content in plant, e.g. beta carotene. The *crtB* gene as disclosed in Proc.Nat..Acad.Sci.USA, 99,1092-1097 can be useful for achieving increased vitamin content, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased starch biosynthesis, e.g. increases sucrose hydrolysis in kernel endosperm that results in increase in starch biosynthesis and endosperm and kernel size. The sucrose phosphorylase, *gftA sp1* gene as disclosed in US patents 6,235,971 and 6,476,295 can be useful for achieving increased starch biosynthesis, e.g. when over expressed in a recombinant DNA construct.

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased endosperm cell number and/or size, e.g. without adverse effects on seed set or germination. See table 9 for genes that can be useful for achieving delayed senescence in plants, e.g. when over expressed in a recombinant DNA construct.

Table 9

Increased endosperm cell number and/or size	Reference
MEDEA	Plant Cell 12, 2367-2381
FIE	PNAS 97(19):10637-42. (2002)
DME	Cell 2002: 110, 33-42.(2002)
MSI1	EMBO J. 22(18):4804-4814. (2003)
ANT	US patent application publication 2002/0170093 A1

Increased endosperm cell number and/or size	Reference
ETR1-1	WO 95/01439; Plant Physiol. 115: 737-751; Plant Mol Biol 44: 283-301.
APL1, APL2, APL3 and <u>APL4</u> fus3/lec mutants,.	Journal of Biological Chemistry 0278: 28508-28515 2003.
	Development 128(2):243-52. (2001)

5 A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased photosynthetic efficiency, e.g. increased total carbon fixation rate in plants under either normal or saturated light and carbon dioxide environments. The *CCA1* gene as disclosed in Cell  
93(7):1207-17 can be useful for achieving increased photosynthetic efficiency, e.g. when over  
expressed in a recombinant DNA construct.

10 A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is increased source enhancement, e.g. as characterized by increased sugar accumulation, increased starch accumulation and/or sucrose export. See table 10 for genes that can be useful for  
achieving delayed senescence in plants, e.g. when over expressed in a recombinant DNA  
construct.

Table 10

Increased source enhancement	Reference
StcPGM (cytosolic isoform of phosphoglucomutase)	Planta (2002) 214:510-520.
SoSUT1	Planta. 2003 May;217(1):158-67.
CCA1	US provisional application No. 60/494397
Sucrose Phosphate Synthase	Planta (2001) 212: 817-822 and numerous external and internal reports
FBPase/SBPase bifunctional	Nature Biotech. 19: 965-969; 2001
<i>VfAGP</i>	Planta 214(6), 954-64 (2002)
ELIP1 (Early light-induced protein1)	PNAS:100; 4921-04926, 2003

Increased source enhancement	Reference
PsbS / NPQ4 Nonphotochemical Quenching 4	PNAS ;99: 15 222-15227, 2002

5 A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is enhanced transport of amino acids, e.g. as characterized by increased accumulation or transport of glutamic acid, glutamine, aspartic acid or asparagine. See table 11 for genes that can be useful for achieving enhanced transport of amino acids, e.g. when over expressed in a recombinant DNA construct.

Table 11

enhanced transport of amino acids	Reference
AAP1	PNAS (1993) 90: 5944-5948
AAP2	Plant Journal (1993) 4:993-1002
AAP3	J Biol Chem (1994) 27: 16315-16320
AAP4	J Biol Chem (1994) 27: 16315-16320
AAP5	J Biol Chem (1994) 27: 16315-16320
AAP6	Plant Cell (1996) 8: 1437-1446
AAP7	J Biol Chem. (2002) 277:45338-45346.
AAP8	J Biol Chem (2002) 277:45338-45346
AUX1	Science (1996) 273: 948-950
ProT	Plant Cell (1996) 8: 1437-1446
LHT1	Plant Physiology (1997) 115: 1127-1134

enhanced transport of amino acids	Reference
ANT1	Plant Physiology (2001) 125: 1813-1820
amino acid transporter	Arabidopsis TxP experiment for G1543 transgenics. PHEN280
Beta-GDH	Internal data

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is enhanced water deficit tolerance, e.g. as characterized by resistance and/or tolerance to water deficit, dehydration resulting from exposure to saline water, freezing conditions, low humidity and/or recovery after water deprivation. See table 12 for genes that can be useful for achieving enhanced water deficit tolerance, e.g. when over expressed in a recombinant DNA construct.

Table 12

Water deficit tolerance	Reference
SOD(Mn superoxide dismutase )	Maria et al., 2002
IMT1 (Myo-inositol o-methyltransferase (D-ononitol synthesis) )	Plant Physiol, 115, (3) 1211-1219 (1997)
CBF4 (Transcription factor)	Plant Physiol.;130(2):639-48 (2002)
mt1D (Mannitol-1-phosphate dehydrogenase (mannitol synthesis))	Plant Physiol. 131(4):1748-55 (2003)
CRE1	The Journal of Cell Biology 161 (6);1035-1040.
NtAQP1 (PIP1 plasma membrane aquaporin)	Plant Cell. 2002 Apr;14(4):869-76
otsA (Trehalose-6-phosphate synthase (trehalose synthesis)	Plant Physiol. 152, 525-532 (1998)
otsB (Trehalose-6-phosphate synthase (trehalose synthesis)	Plant Physiol. 152, 525-532 (1998)

Water deficit tolerance	Reference
<i>NADPH-dependent aldose/aldehyde reductase</i>	Plant J.24(4):437-46 (2000)
PLD alpha (Phospholipase D (alpha) expression )	Plant J. 2001 Oct;28(2):135-44
OsCDPK7(Transcription factor )	Plant J. 2000 Aug;23(3):319-27
Transcription factors	US provisional application serial No. 60/449054, incorporated herein by reference
GB1	US provisional application serial No.60/467910 and 60/487273, all incorporated herein by reference
SOD	Plant Physiol. 1996 Aug;111(4):1177-1181
BADH-1 (Betaine aldehyde dehydrogenase )	Soil Science and Plant Nutrition ,46 (4), 873 - 884 (2000)
Adc (Arginine decarboxylase )	Capell et al. 1998
ME (NADP-malic enzyme which converts malate and NADP to pyruvate, NADPH, and CO <sub>2</sub> )	Laporte et al. 2002
AtGolS2 (Galactinol and raffinose accumulation)	Plant J. ;29(4):417-26. (2002)
AtNCED3 (Increased ABA synthesis)	Plant J. 2001 Aug;27(4):325-33
AtHAL3a (Phosphoprotein phosphatase)	Plant J.20, 529-539 (1999)
Apo-Inv ( Apoplastic invertase )	Plant and Cell Physiology, 42, (2) 245-249
NPH1/PHOT1	NATURE, 414: 656:660
GPA1	Science 292,2070-2072

A useful crop improvement trait for stacking with herbicide and/or pest resistance traits is enhanced heat tolerance. See table 13 for genes that can be useful for achieving enhanced heat tolerance, e.g. when over expressed in a recombinant DNA construct.

5



Table 13

heat tolerance	Reference
Apx1	Gene. 2001 Jul 25;273(1):23-7
Hsp101	Plant Mol Biol. 2003 Mar;51(4):543-53
<i>AtHSP101</i>	PMB 51, 677-686, 2003
<i>ChyB</i>	Nature 418(6894):203-6 (2002)
hs	Plant J. 1995 Oct;8(4):603-12
TLHS1	Mol Cells. 31; 10(5):519-24

5           **Transformation Methods and Transgenic Plants** - Methods and compositions for transforming plants by introducing a recombinant DNA construct into a plant genome in the practice of this invention can include any of the well-known and demonstrated methods. Preferred methods of plant transformation are microprojectile bombardment as illustrated in U.S. Patents 5,015,580; 5,550,318; 5,538,880; 6,160,208; 6,399,861 and 6,403,865 and

10 *Agrobacterium*-mediated transformation as illustrated in U.S. Patents 5,635,055; 5,824,877; 5,591,616; 5,981,840 and 6,384,301, all of which are incorporated herein by reference. See also U.S. application Serial No. 09/823,676, incorporated herein by reference, for a description of vectors, transformation methods, and production of transformed *Arabidopsis thaliana* plants where transcription factors are constitutively expressed by a CaMV35S promoter.

15           Transformation methods of this invention to provide plants with enhanced environmental stress tolerance are preferably practiced in tissue culture on media and in a controlled environment. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. Recipient cell targets include, but are not limited to, meristem cells, callus, immature embryos and gametic cells such as microspores,

20 pollen, sperm and egg cells. It is contemplated that any cell from which a fertile plant may be regenerated is useful as a recipient cell. Callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the like. Those cells which are capable of proliferating as callus also are recipient cells for genetic transformation. Practical transformation methods and materials for making transgenic plants of

25 this invention, e.g. various media and recipient target cells, transformation of immature embryos

and subsequent regeneration of fertile transgenic plants are disclosed in U.S. Patents 6,194,636 and 6,232,526 and U.S. patent application Serial No. 09/757,089, which are incorporated herein by reference.

The seeds of this invention can be harvested from fertile transgenic plants and be used to grow progeny generations of transformed plants of this invention including hybrid plants line comprising the DNA construct expressing a transcription factor which provides the benefits of resistance and/or tolerance to water deficit.

Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration, and is not intended to be limiting of the present invention, unless specified. These examples illustrates the use of polynucleotides encoding a water-deficit tolerance-imparting transcription factor to provide various transgenic plants exhibiting enhanced tolerance for and/or resistance to growing conditions of water deficit.

#### **Breeding of Transgenic Plants**

In addition to direct transformation of a plant with a recombinant DNA construct, transgenic plants can be prepared by crossing a first plant having a recombinant DNA construct with a second plant lacking the construct. For example, recombinant DNA can be introduced into a plant line that is amenable to transformation to produce a transgenic plant which can be crossed with a second plant line to introgress the recombinant DNA into the second plant line.

In one aspect of the invention a transgenic plant with recombinant DNA conferring a crop improvement trait is crossed with a transgenic plant having recombinant DNA conferring herbicide and/or pest resistance to produce progeny plants having recombinant DNA that confers both the crop improvement trait and the herbicide and/or pest resistance trait. Preferably, in such breeding for combining traits the transgenic plant donating the crop improvement trait is a female line and the transgenic plant donating the herbicide and/or pest resistance trait is a male line. The progeny of this cross will segregate such that some of the plant will carry the DNA for both parental traits and some will carry DNA for one parental trait; such plants can be identified by markers associated with parental recombinant DNA. Progeny plants carrying DNA for both parental traits can be crossed back into the female parent line multiple times, e.g. usually 6 to 8 generations, to produce a progeny plant with substantially the same genotype as one original transgenic parental line but for the recombinant DNA of the other transgenic parental line.

In yet another aspect of the invention hybrid transgenic seed, e.g. a hybrid transgenic corn seed, is produced by crossing a female transgenic corn line containing recombinant DNA conferring a crop improvement trait with a male transgenic corn line containing recombinant DNA conferring herbicide and/or pest resistance. In a preferred aspect of this invention hybrid transgenic corn seed is produced by crossing a female transgenic corn line with recombinant DNA conferring both a crop improvement trait and herbicide resistance with a male transgenic corn line with recombinant DNA conferring both herbicide resistance and pest resistance.

### Example 1

This example illustrates aspects of the invention through the production of transgenic corn plants and seed with recombinant DNA that confers water-deficit tolerance.

Transgenic corn was transformed with a recombinant DNA construct having the nucleotide sequence of SEQ ID NO:1 and which comprises a rice actin 1 constitutive promoter and a rice actin 1 intron operably linked an corn gene which encodes a Hap3 transcription factor with the amino acid sequence of SEQ ID NO:2 followed by a Tr7 3' terminator. The construct further comprised a CaMV 35 S promoter operably linked to an nptII marker gene. Twenty transgenic events in corn were selected as having either 1 or 2 copies of the construct and no oriV origin of replication from the vector. Eleven of the twenty events survived to fertile plants which produced seed. Seed producing plants were analyzed to verify the presence of the exogenous DNA encoding the transcription factor and accumulation of the transcription factor. Six of the transgenic events were used in a water deficit assay.

Pre-germinated seedlings of transgenic plants (progeny of a heterozygous transgenic plant which inherited the exogenous transcription factor DNA construct) and wild type plants (progeny of a heterozygous transgenic plant which did not inherit the exogenous transcription factor DNA construct) were planted in 5 inch pots containing 330 grams of soil. The plants were well watered for one week then allowed to dry for 4 days. An equal number (32) of transgenic and wild type plants were selected based on matched height and the selected plants were mixed in eight flats. Four flats were designated as "wet" meaning they would be well watered and four flats were designated as "dry" meaning they would be subject to water deficit. All pots were brought to the weight of the heaviest pot by adding water. The pots were weighed daily until the average pot weight dropped to between 600 to 700 grams, whereupon a water deficit assay was

started by measuring plant heights and resuming watering for pots in “wet” flats while continuing to withhold water for pots in “dry” flats.

5 The pots in the “wet” flats were fully-watered daily. The pots in the “dry” flats were weighed daily to determine a water deficit treatment. If the average “dry” flat pot weight was greater than 500 grams, no water was added; if the average pot weight was between 365 and 500  
grams, 35 grams of water was added to each pot; and, if the average pot weight was less than 365 grams, a determined amount of water was added to bring the average pot weight to 400 grams. The water deficit treatment was continued until the pots in the “dry” flats have had an average  
10 pot weight below 500 grams for 8 days. The height of all plants was measured on the 9<sup>th</sup> day and averages are reported in Table 1.

Table 1  
Average Change in Plant Height (cm) through first water deficit

Treatment	Transgenic	Wild Type	Ratio	Significance
Full water	46.3 cm	45.5 cm	1.02	0.03
Water deficit	20.5 cm	19.7 cm	1.04	0.015

On the 9<sup>th</sup> day full watering was resumed for the “dry” flat pots for 3 days when heights were again measured. See tables 2 and 3 for changes in average plant height for the eight water-deficient plants which were recovered during the 3-day recovery period. Table 2 shows the average incremental change in plant height for the recovered plants which occurred during the 3-day recovery period. Table 3 show the total average change in plant height for the recovered plants which occurred through the combined water-deficit and recovery periods.

Table 2  
Average Change in Recovered Plant Height During 3-day Recovery Period

Treatment	Transgenic	Wild Type	Ratio	Significance
Water recovered	13.2 cm	13.2 cm	1.0	0.74

Table 3  
Average Change in Recovered Plant Height Through Deficit and Recovery

Treatment	Transgenic	Wild Type	Ratio	Significance
Water deficit	33.8 cm	32.9 cm	1.03	0.03

Recovered plants were subjected to a second round of water deficit as described above. After 9 days full water was resumed for 7 days. After two days of full water the twice water-deficient plants began to show signs of recovery from a wilted state. In some cases recovery took 5-6 days and some plants never recovered. On average the recovered transgenic plants were significantly greener and healthier than recovered wild type plants which were more wilted and yellow (indicating senescence).

### Example 2

This example further illustrates the aspect of this invention relating to transgenic corn. Progeny seed of the transgenic corn produced in Example 1 was planted in a field trial to evaluate its water deficit tolerance as compared to the negative sibling (wild type). The plants were grown in a well irrigated field in Kansas. Water was withheld from half of the planting during the late vegetative stage. The experimental evidence showed that under water deficit conditions transgenic corn plants expressing the Hap3 transcription factor of SEQ ID NO:2 were healthier than the wild type and exhibited the following phenotypes:

- (a) likely to have a higher chlorophyll index, e.g. >42 in transgenic plants as compared to <40 in wild type,
- (b) likely to produce more photosynthate,
- (c) likely to have cooler leaf temperature, and
- (d) likely to maintain higher stomatal conductance.

### Example 3

This example illustrates the invention through the preparation of transgenic seed and plants with a crop improvement trait, e.g. water-deficit tolerant soybean.

Transgenic soybean was transformed with a recombinant DNA construct comprising a CaMV 35S constitutive promoter operably linked to an *Arabidopsis thaliana* gene which encodes the Hap3 transcription factor having the amino acid sequence of SEQ ID NO:7 followed by a terminator element. In a water deficit assay Transgenic soybean plants exhibited enhanced resistance to water deficit, i.e. less wilting, as compared to wild type soybean plants. In addition In particular, transgenic plants wilted less, had a higher chlorophyll content, had a higher relative water content, had a higher photosynthesis rate, than their gene negative segregants and parental control plants.

### Example 4

This example illustrates the invention through the preparation of transgenic seed and plants with a crop improvement trait, e.g. water-deficit tolerant soybean.

Transgenic soybean was transformed with a recombinant DNA construct comprising a CaMV 35S constitutive promoter operably linked to an endogenous soybean gene having the

nucleotide sequence of SEQ ID NO:5 which encodes the native Hap3 transcription factor having the amino acid sequence of SEQ ID NO:6 followed by a terminator element. In a water deficit assay where water was withheld after saturating potted plants at the V1 stage until the soil reached 10% of capacity (50% for well watered control), transgenic soybean plants exhibited enhanced resistance to water deficit as compared to wild type soybean plants. In particular, transgenic plants wilted less, had a higher chlorophyll content, had a higher relative water content, had a higher photosynthesis rate, than their gene negative segregants and parental control plants.

### Example 5

This example illustrates aspects of the invention through the preparation of transgenic seed and plants with a crop improvement trait and herbicide and insect resistance traits.

Transgenic maize is transformed with recombinant DNA constructs substantially as disclosed in Example 1 except that the selective marker is an EPSPS gene that confers resistance to glyphosate herbicide. The transgenic plants produce seed which can be used to grow water-deficit-tolerant progeny plant which can be bred with transgenic plants having pest resistance to provide progeny plants with stacked engineering traits.

Seed from plants with water deficit tolerance and glyphosate herbicide tolerance are used as female plants in breeding with a pollen from transgenic plants with insect resistance, e.g. maize line MON863 available from Monsanto Company, St. Louis, MO, which contains recombinant DNA expressing the *cry3Bb1* gene encoding a Coleopteran-specific insecticidal protein from *Bacillus thuringiensis* (subsp. *kumamotoensis*) to control infestation with corn root worm (CRW; *Diabrotica sp.*). Segregating progeny plants are selected for all three traits, i.e. water deficit tolerance, herbicide tolerance and insect resistance. Selected plants are back crossed for 6 generations with the water deficit tolerant line. By such breeding the insect resistance trait is introgressed into the transgenic line with water deficit tolerance and glyphosate herbicide tolerance.

### Example 6

This example illustrates another aspect of the invention through the preparation of transgenic seed and plants with a crop improvement trait and herbicide tolerance and insect resistance traits.

Transgenic maize is transformed with recombinant DNA constructs substantially as disclosed in Example 1 except that the selective marker is an *bar* gene that confers tolerance to glufosinate herbicide. Seed from water deficit-tolerant, glufosinate herbicide-tolerant plants were used as female plants in breeding with a pollen from a glyphosate herbicide-tolerant, insect-resistant transgenic corn plants, e.g. maize line MON802 available from Monsanto Company, St. Louis, MO and which has recombinant genes encoding the CryIAb protein from *Bacillus thuringiensis* and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *A. tumefaciens* strain CP4. Segregating progeny plants are selected for water deficit tolerance by screening with glufosinate herbicide and insect resistance by screening with glyphosate herbicide.



We claim:

1. A seed for growing an herbicide-resistant, transgenic plant with a crop improvement trait produced by inserting into the genome of a progenitor plant a recombinant DNA construct comprising DNA which confers at least one crop improvement trait and at least one of an herbicide resistance trait or a pest resistance trait.
2. A seed of claim 1 produced by inserting into the genome of a progenitor plant a recombinant DNA construct comprising DNA which confers at least one crop improvement trait and an herbicide resistance trait.
3. A seed of claim 2 wherein said crop improvement trait is selected from the group consisting of cold tolerance, improved nitrogen processing, improved phosphorus use efficiency, reduced abscission, early maturity, enhanced growth and leaf development, enhanced growth rate, low light tolerance, optimized C3 to C4 transition, improved plant development, increased plant size, population stress tolerance, reduced plant size, improved seed germination, delayed senescence, increased amino acid content, increased seed protein, increased seed oil, increased seed starch biosynthesis, increased endosperm cell number and/or size, increased vitamin content, increased photosynthesis efficiency, increased source enhancement, enhanced transport of amino acids and enhanced water deficit tolerance.
4. A seed of claim 3 wherein said herbicide resistance trait is characterized as resistance to an herbicide selected from the group consisting of a glyphosate herbicide, a phosphinothricin herbicide, an oxynil herbicide, an imidazolinone herbicide, a dinitroaniline herbicide, a pyridine herbicide, a sulfonylurea herbicide, a bialaphos herbicide, a sulfonamide herbicide and a gluphosinate herbicide.
5. A seed of claim 4 wherein said crop improvement trait is water deficit tolerance conferred by a recombinant DNA construct comprising a gene which encodes a Hap3 protein having a consensus amino acid sequence of SEQ ID NO:10.
6. A seed of claim 5 having in its genome a recombinant DNA construct which confers pest resistance through expression of a gene which encodes an insecticidal protein.
7. A seed of claim 6 wherein said insecticidal protein is a delta endotoxin from *Bacillus thuringiensis*.
8. A seed of claim 5 having in its genome a recombinant DNA construct which transcribes RNA which forms gene silencing dsRNA targeted to a crop pest.

9. A seed of claim 5 which can be grown to produce a soybean plant which is resistant to glyphosate herbicide.
10. A seed of claim 5 which can be grown to produce a corn plant which is resistant to glyphosate herbicide.
- 5 11. A seed of claim 5 which can be grown to produce a cotton plant which is resistant to glyphosate herbicide.
12. A seed of claim 5 which can be grown to produce a canola plant which is resistant to glyphosate herbicide.
13. A seed of claim 5 which can be grown to produce a wheat plant which is resistant to  
10 glyphosate herbicide.
14. A hybrid corn seed which is the progeny of
  - (a) a transgenic female ancestor corn plant having in its genome a recombinant DNA which confers a crop improvement trait;
  - (b) a transgenic male ancestor corn plant having in its genome a recombinant DNA which  
15 confers at least one of an herbicide resistance trait or a pest resistance trait.
15. A hybrid corn seed of claim 14 wherein said transgenic female ancestor corn plant further has in its genome recombinant DNA which confers herbicide resistance.
16. A hybrid corn seed of claim 14 wherein said transgenic male ancestor corn plant has in its genome recombinant DNA which confers both herbicide resistance and insect resistance.
- 20 17. A seed of claim 14 wherein said crop improvement trait is selected from the group consisting of cold tolerance, improved nitrogen processing, improved phosphorus use efficiency, reduced abscission, early maturity, enhanced growth and leaf development, enhanced growth rate, low light tolerance, optimized C3 to C4 transition, improved plant development, increased plant size, population stress tolerance, reduced plant size, improved seed germination, delayed  
25 senescence, increased amino acid content, increased seed protein, increased seed oil, increased seed starch biosynthesis, increased endosperm cell number and/or size, increased vitamin content, increased photosynthesis efficiency, increased source enhancement, enhanced transport of amino acids and enhanced water deficit tolerance
18. A hybrid corn seed of claim 17 wherein said crop improvement trait is trait is water  
30 deficit tolerance conferred by a recombinant DNA construct comprising a gene which encodes a Hap3 protein having a consensus amino acid sequence of SEQ ID NO:10.

19. A hybrid corn seed of claim 18 having resistance to at least one herbicide selected from the group consisting of a glyphosate herbicide, a phosphinothricin herbicide, an oxynil herbicide, an imidazolinone herbicide, a dinitroaniline herbicide, a pyridine herbicide, a sulfonylurea herbicide, a bialaphos herbicide, a sulfonamide herbicide and a gluphosinate herbicide..

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20. A hybrid corn seed of claim 18 having resistance to at least two herbicides selected from the group consisting of a glyphosate herbicide, a phosphinothricin herbicide, an oxynil herbicide, an imidazolinone herbicide, a dinitroaniline herbicide, a pyridine herbicide, a sulfonylurea herbicide, a bialaphos herbicide, a sulfonamide herbicide and a gluphosinate herbicide..

10

FIGURE 1  
Amino Acid Sequence Alignment of CCAAT Box DNA Binding Transcription Factors

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SEQ ID NO:7 MADTPSSPAG DGG---ES-- -----GGSVR EQDRYLPIAN ISRIMKKALP PNGK-----
SEQ ID NO:8
SEQ ID NO:10MXXXXPXPXX
R EQDRYLPIAN ISRIMKKALP PNGKXXXXXX

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&lt;141&gt; 2003-10-02

&lt;160&gt; 10

&lt;170&gt; PatentIn version 3.2

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